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Quantitative Analysis of the T Cell Repertoire that Escapes Negative Selection

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Summary

Mice expressing hen egg-white lysozyme (HEL) as a transgene are unresponsive to immunization with the HEL protein. Profound tolerance was found even in situations where the amounts of I-A^k-peptide complexes was 100 or less per APC. Among the few T cells that escaped tolerance, we did not observe differential responses to the different HEL epitopes, perhaps because of the very high sensitivity of the negative selection process. The same HEL transgenic mice that did not respond to HEL responded to immunization with the 46–61 peptide of HEL. These peptide-specific T cells that escaped negative selection belonged to a set that reacted with a particular conformer of the HEL peptide-I-A^k (type B). The presence of type B reactive T cells should be considered in autoimmunity.

Introduction

This paper examines tolerance to the protein antigen hen egg-white lysozyme (HEL). We evaluated the response to HEL in the context of information now available concerning the peptides recognized by T cells in inbred mice bearing the class II molecule I-A^k. HEL induces strong B cell and CD4 T cell responses to several of its epitopes, some of which have been well characterized (Allen and Unanue, 1984; Adorini et al., 1988; Goodnow et al., 1988; Gammon et al., 1991).

Our laboratory has isolated several peptide families from I-A^k molecules of antigen-presenting cells (APCs) after the processing of HEL: the predominant peptide family encompassed a nested series centered on the core sequence 52–61. The majority of the peptides were from residues 48 extending to residues 61, 62, or 63 (i.e., DGSTDYGILQINSR/W/W). For convenience we will refer to this family as 48–62 (Nelson et al., 1992). We have also described another family of peptides starting from residue 31 or 32 and ending with residues 46–49, presented at about 20-fold lower levels than the 48–62 epitope (Gugasyan et al., 1998). Importantly for this study, we also have identified two putative conformers of the same linear peptide 48–61-I-A^k complex (Viner et al., 1996). The type A conformer was produced after HEL was processed by APCs and the 48–62 epitope was selected by I-A^k molecules (endogenous pathway). The type B conformer was produced when the APCs were pulsed with the same peptide (exogenous pathway). It is important to note that type B–specific T cells recognized the same linear sequences bound to I-A^k as type A–specific T cells, but recognition by type B T cells was restricted to a conformer dependent on the manner in which the peptide was loaded. Type A–specific T cells did not distinguish between these conformations, recognizing the peptide-I-A^k complex loaded endogenously or exogenously. Table 1 offers a comparison of these two different epitopes generated from the same linear sequence. Recent findings have confirmed that the formation of multiple conformations of a single peptide-MHC complex can be observed in vitro (Rabinowitz et al., 1997; Gyotoku et al., 1998). A prediction from the finding of these two conformers is that some T cells directed to a type B conformer of a self-peptide-MHC complex may escape negative selection when HEL is presented as an autologous antigen by thymic APCs. These T cells could conceivably then have the potential to react in peripheral tissues in conditions of inflammation that generate the peptide from the autologous antigen.

The issue of differential sensitivity to tolerance of T cell epitopes has been discussed previously (Gammon and Sercarz, 1989; Milich et al., 1989, 1991; Cibotti et al., 1992; Fairchild et al., 1993; Joosten et al., 1994; Cerasoli et al., 1995; Gapin et al., 1997; Shih et al., 1997; Harrington et al., 1998). An epitope may be highly tolerogenic because of a high level of expression by MHC molecules, perhaps reflecting its high binding affinity for a class II molecule. In contrast, a second epitope may be expressed at very limiting amounts because it binds weakly, failing to induce negative selection (Gammon and Sercarz, 1989). This latter situation may be represented by the various myelin basic protein epitopes responsible for disease in the experimental allergic encephalitis (EAE) model of multiple sclerosis (Fairchild et al., 1993; Joosten et al., 1994; Harrington et al., 1998). Our own laboratory has observed that poor central tolerance may be a feature distinguishing the class II molecule I-A^{g7} of the NOD mouse model of type I diabetes (Carrasco-Marin et al., 1996; Kanagawa et al., 1998).

Knowing the densities of the HEL epitopes involved in the T cell response, we attempt here to make some quantitative estimates of the sensitivity of negative selection when HEL is expressed as a transgene in APCs. We will show here that thymic tolerance or negative selection operates at the level of a few peptide-MHC complexes. This could be expected based on various approaches (Yagi and Janeway, 1990; Pircher et al., 1991; Vasquez et al., 1992; Sebzda et al., 1994; Kimachi et al., 1997; Peterson et al., 1999). We will also show that some T cells that recognize the B conformers escape negative selection and peripheralize. Our experiments were done by quantitating the number of peptide-reactive T cells by a very sensitive limiting dilution analysis (LDA).

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Table 1. A and B Conformations of HEL Peptide 48–62

APC	Antigen	A	B
(1) Live	HEL	+	−
(2) Live	membrane HEL	+	−
(3) Live	denatured HEL	+	+
(4) Live	synthetic peptide 48–62	+	+
(5) Live	tryptic peptide 46–61	+	+
(6) Live	covalently-linked 48–62	+	−
(7) Live	I-A^k extracted 48–62	+	+
(8) Fixed	HEL	−	−
(9) Fixed	48–62	+	+
(10) Fixed	denatured HEL	+	+
(11) I-A^k purified	48–62	+	+

This table summarizes the findings from our laboratory on the two kinds of 48–62-reactive states (Viner et al., 1996; Latek and Unanue, 1999; DiPoalo and Unanue, unpublished data). The A conformer is produced by processing of HEL (numbers 1 and 2, above); importantly, 48–62 engineered on I-A β^k chain, following the study of Kappler and Marrack's laboratory (Ignatowicz et al., 1995) induces the A conformer but not the B (6). Type B conformer is produced when either peptide (4, 5, 7, and 9) or denatured protein (3 and 10) are added to live or fixed APCs. The use of fixed APC indicates that the peptide bound to I-A^k triggers the B-reactive T cell, an indication that the peptide does not require further internalization or processing to yield the B state. The use of tryptic peptide, in (5), indicates that the conformer is not the consequence of modifications in the synthetic peptide. Also note that 48–62 extracted from I-A^k after processing (1) stimulates the cells reactive with the B conformer (7); i.e., the same peptide not recognized during HEL processing is recognized after being offered directly to the APC.

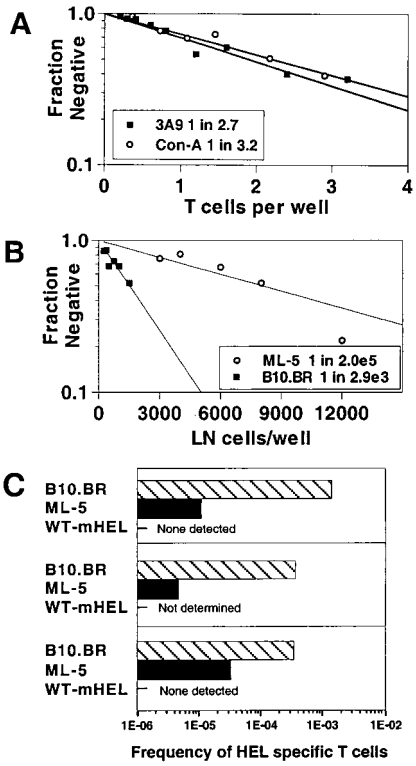

Figure 1. Limiting Dilution Analysis of Normal and HEL Transgenic Mice

(A) Limiting dilution analysis (LDA) of T cells from either a 3A9 transgenic mouse or B10.BR mouse was determined using either HEL or concanavalin A (Con A) as the stimulatory antigen in vitro. The fraction of growth-negative wells (as determined by visual inspection under an inverted microscope) was plotted as a function of the number of T cells added per well as determined by FACS. The fraction of lymph node cells from 3A9 mice positive for the clonotype was 16% as determined by FACS. The fraction of lymph node cells from B10.BR mice positive for CD4 or CD8 markers was 29%. The frequencies from the LDA were derived as described in the Experimental Procedures.

(B) LDA of B10.BR and ML-5 transgenic mice immunized with 10 nmols HEL demonstrated tolerance in ML-5 mice. The fraction of negative wells was plotted as a function of the number of cells added per well. Positive wells were identified by growth, and subsequent HEL specificity was determined. The frequencies displayed were corrected for specificity. Each draining lymph node contained roughly 10^7 total cells. Twelve to seventeen percent of lymph node cells were $CD4^+$ by flow cytometry; the number of CD4 cells was similar for all strains of mice in individual experiments. These results are representative of the one of the three experiments in Figure 2C (middle panel). There was a 76-fold decrease in HEL specific precursors in the ML-5 mice.

(C) T cell tolerance observed in both WT-mHEL and ML-5 mice immunized with HEL. Precursor frequency was determined by LDA followed by specificity analysis. The precursor frequency for HEL immunized mice is expressed on the x axis. For example, a precursor frequency of 1 in 1000 represents $1e^{-3}$ as plotted here.

Results

Response of Normal Mice to HEL Immunization

Mice were immunized with a dose of HEL that optimally elicits a T cell response. We evaluated the T cell response with an LDA that identifies one of every two to three responding T cells (Kelso and Glasebrook, 1984; Kanagawa et al., 1998). (This sensitivity was tested using either the response of the 3A9 T cell receptor [TCR] transgenic mice to HEL or the response of T cells from B10.BR mice to concanavalin A [Figure 1A].) Following immunization, each HEL-specific T cell in the analysis was expanded and tested against various HEL peptides found to elicit a T cell response. The peptides tested are shown in Figure 2, along with their binding strength for I-A^k. It is notable that only the 48–63 and 31–49 sequences correspond to those of the natural peptide (i.e., the peptides selected by I-A^k during processing of HEL in APC). Whether the other peptides used correspond exactly to their natural sequences has yet to be determined.

The draining lymph nodes of B10.BR mice immunized with HEL contained 1 per 750 to 1 per 3700 HEL-specific T cells (Figures 1B and 1C). The precursor frequency is displayed by the bars in Figure 1C where a frequency of 1 in 1000 would be equal to $1e^{-3}$ as shown on the x axis. In 337 clones examined, the predominant response was to epitopes 48–63 (31% of the clones), 18–33 (32%), and 115–129 (21%) (Figure 2). The 31–49 epitope was only 7% of the I-A^k-restricted response. Of the I-A^k-restricted response, the specificity of 91% of clones was determined. The remaining 9% included the epitopes 70–88 and 98–112, which were not examined for all experiments. When examined, we found that the response to epitopes 70–88 and 98–112 represented 4% and 1%, respectively (data not shown). Of the total responding T cells, 12% was restricted by I-E^k. The epitopes for these T cells were not examined.

The repertoire of HEL-specific T cells in the B10.BR may reflect the impact of the expression of the mouse homolog murine lysozyme. This protein is expressed

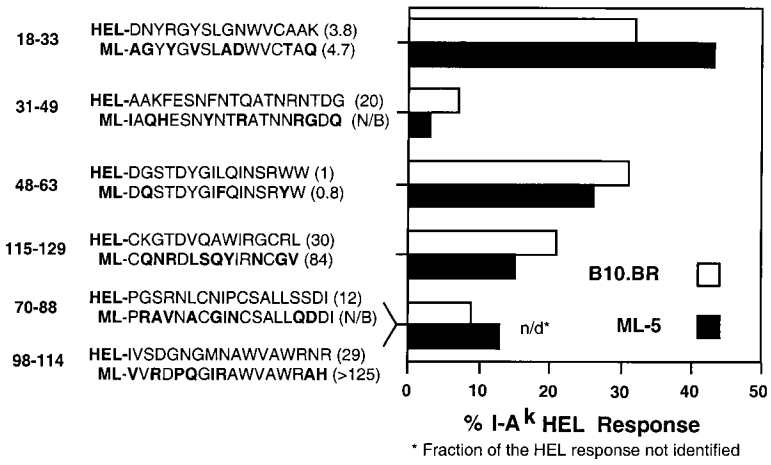

Figure 2. The Peptide Specificity of the HEL-Specific T Cells in the B10.BR and ML-5 Mice

The bars depict the percent of the I-A^k-restricted HEL response specific for each of the four major epitopes of HEL. The sequence of HEL and mouse lysozyme M (ML) peptide are displayed next to their respective bars (amino acid differences are highlighted in bold). The RIC^{-1} values, shown in parentheses, represents the relative concentration of the peptide required to compete for binding of detergent-soluble I-A^k by ^{125}I-labeled reference peptide, as described previously (Nelson et al., 1996). Epitopes 70–88 and 98–114 were *not* tested in every experiment. These are displayed next to the fraction of response not specific for the four predominant peptides. While there was a clear pattern of T cell response, there was no major difference in the T cell repertoire of ML-5 (solid bars) compared to B10.BR (open bars). The repertoire of B10.BR and ML-5 was determined by the identification of 337 and 132 I-A^k-restricted T cells, respectively. Twelve percent of the B10.BR response and 15% of the ML-5 response were I-E^k restricted.

normally in some APCs. Thus, it was important to examine the murine lysozyme peptides in terms of both their degree of homology to HEL peptides as well as their binding strength for I-A^k molecules. Most murine peptides exhibit significant amino acid differences with the corresponding HEL sequences.

The impact of murine lysozyme epitopes on the HEL repertoire was difficult to observe (Figure 2). Moudgil and Sercarz had predicted that epitopes of murine lysozyme that are not presented would correspond to HEL-specific T cells repertoire in the nontransgenic response (Moudgil and Sercarz, 1993). However, this relationship did not hold in these experiments. Of the murine lysozyme peptides, 31–49 and 70–88 failed to bind I-A^k, and yet they were poorly represented in the corresponding HEL-specific T cell repertoire (Figure 2). Moreover, the 48–63 murine peptide bound to I-A^k as well as the corresponding HEL peptide, yet this was a major epitope in the B10.BR repertoire (Figure 2).

In summary, the binding strength of the HEL epitopes for I-A^k does not explain the distribution of the HEL-specific T cell repertoire (i.e., comparing 48–63 and 115–129 in Figure 3). The repertoire for foreign antigen must therefore reflect a complex mixture of multiple parameters, of which we have only analyzed two (the impact of binding and the impact of autologous protein).

Response of HEL Transgenic Mice to HEL Immunization

We examined two lines of transgenic mice expressing HEL. The wild-type HEL mice ("WT-mHEL") express a membrane-bound form of HEL on all APCs. HEL-expressing cells were found in both the thymic cortex and medulla (data not shown). APCs of the WT-mHEL mice, harvested from the peritoneal cavity or the spleen, stimulated the 3A9 T cells that recognizes the 48–62 peptide family (Figure 3A). Using the AW3.18 monoclonal antibody specific for the 48–62-I-A^k complex, we quantitated a range of 3,400–20,000 peptide-MHC complexes per APC in the WT-mHEL mice (Dadaglio et al., 1997) (Figure 3B; data not shown).

The "ML-5" mice, originally described by Goodnow et al., were found to exhibit substantial tolerance of the HEL-specific T cells (Goodnow et al., 1988; Adelstein et al., 1991). HEL is produced as soluble protein under the metallothionein promoter, circulating at about 10–20 ng per milliliter of serum. It was previously reported by Kanost et al. that the APCs of the ML-5 mice were unable to directly present the 48–62 epitope to T cells (Kanost et al., 1993). We observed very similar results (Figure 3A), as the ML-5 APCs poorly stimulated the 3A9 T cells. We were unable to reproducibly detect the I-A^k-48–62 complex using the AW3.18 monoclonal antibody. Based on the sensitivity of the assays used, we estimated that the number of complexes of I-A^k-48–62 must be less than 100 per APC.

After immunization of WT-mHEL mice with HEL, we were unable to detect any HEL-specific T cells (Figure 1C). We detected a reproducible number of autoreactive (not HEL-specific) T cells at a frequency between 1 per 4×10^4 and 10^5 cells. In a representative experiment we examined 156 growth-positive wells, 60 of which responded to B10.BR APC in the absence of HEL, while the remaining 96 did not respond. (If 1 out of 156 growth-positive wells had been HEL specific, it would have translated into a frequency of 1 per 6×10^6 cells [$1.6e^{-7}$], out of lymph nodes having $\sim 10^7$ total cells per node.) Lastly, repeated attempts to isolate and propagate HEL-reactive T cells from bulk cultures consistently failed. Again, the only T cells that grew from such cultures were autoreactive but not HEL specific. Therefore, we concluded that tolerance to all HEL epitopes in WT-mHEL mice was complete.

In contrast to the WT-mHEL mice, we identified HEL-specific T cells in the ML-5 transgenic mice immunized with HEL, although at a much reduced frequency when compared to the B10.BR response (Figure 1B). This difference reflects the difference in HEL levels between the transgenic mice (Figure 3A). The precursor frequency of HEL-reactive T cells was 77- to 127-fold lower than in the immunized B10.BR mice, demonstrating incomplete tolerance.

The specificity of the T cells from immunized ML-5 mice did not differ from the immunized B10.BR mice

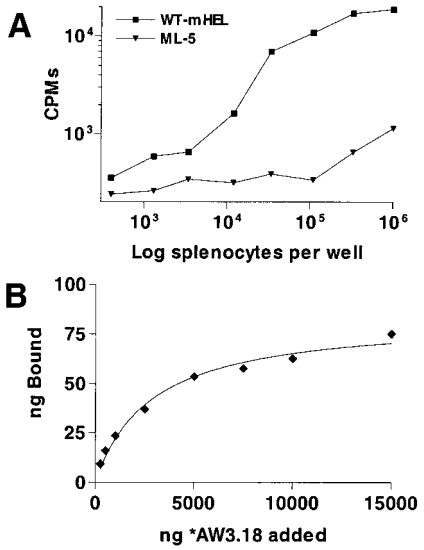

Figure 3. Analysis of Antigen of Presentation in HEL Transgenic Mice

(A) Stimulation of 3A9 transgenic T cells by splenocytes from HEL transgenic mice. Lymph node cells from 3A9 transgenic mice were incubated with titrating numbers of splenocytes from WT-mHEL mice or ML-5 mice. Proliferation of the T cells was measured by [^{3}H]thymidine incorporation. Cultures were pulsed for the last 12 hr of 72 hr cultures. There was roughly a 100-fold difference between WT-mHEL and ML-5 APCs.

(B) Specific binding of the AW3.18 monoclonal antibody to splenocytes of the WT-mHEL mice. T cell–depleted splenocytes (2×10^7) from the WT-mHEL mice were incubated with ^{125}I-labeled AW3.18 monoclonal antibody specific for the 48–62-I-A^k complex. Bound and unbound antibody was separated by spinning the APCs through oil. Nonlinear regression of the antibody-bound versus antibody-added antibody revealed a maximum binding of 83.9 ng/2×10^7 APCs (as described in the Experimental Procedures). In this experiment, this represents 16,800 complexes per APC; Scatchard analysis of the same data placed this value at 15,000 complexes per APC. Results from multiple experiments have ranged from 3,400 to 20,000 complexes per APC.

(Figure 2): neither the strong nor the weak epitopes were preferentially tolerized or spared. The two most prevalent epitopes in the B10.BR response (18–33 and 48–63) were the most prevalent in the ML-5 HEL-specific response. Likewise, the weakest epitope, 31–49, was weakest in the ML-5 HEL response. Apparently all epitopes of HEL were presented in the thymic APCs of the ML-5 mice but not at levels sufficient for complete tolerance.

Development of the 3A9 TCR Transgenic T Cells in HEL Transgenic Mice

To examine further the sensitivity of the negative selection process, we evaluated the development of T cells in the 3A9 TCR transgenic mice crossed to the different HEL transgenic mice (Ho et al., 1994; Akkaraju et al., 1997). Akkaraju et al. (1997) studied the development of 3A9 T cells in mice bearing the 3A9 transgene crossed to the ML-5 or a membrane HEL transgenic mice (under a class I MHC promoter), both in the H-2^{kxb} F1 haplotype. In their results, they observed incomplete deletion of 3A9 expressing T cells by the ML-5 mice. We observed that the two HEL transgenic mice (WT-mHEL and ML-5) completely deleted the 3A9 idiotype containing T cells, which were also absent from spleen and lymph nodes. These two transgenic lines differed in the extent of thymocyte deletion. The ML-5/3A9 double-transgenic mice had almost 10-fold fewer thymocytes than the 3A9 control transgenic mice. The deletion, however, was more marked in the WT-mHEL/3A9 double-transgenic mice—about 100-fold reduction in total thymocytes compared the 3A9 control transgenic mice (Figure 4). In addition there was a small population of CD4 and CD8 double-positive thymocytes that persisted in the ML-5/3A9 double-transgenic mice, while the WT-mHEL/3A9 double-transgenic mice had none. These results indicate that negative selection was occurring on the low number of complexes on the APCs of the ML-5 mice. The differences between our results and those of Akkaraju et al. may reflect either a lower level of presentation of the 48–62 epitope or inefficient positive selection on the H-2^{kxb} background (Tourne et al., 1999).

Response of HEL Transgenic Mice to Peptide Immunization

We analyzed the response of the HEL transgenic mice to immunization with the dominant 48–62 epitope. The mice were immunized with the natural tryptic peptide 46–61 from HEL in order to avoid any response to contaminants found in synthetic peptides (Schild et al., 1991; Purcell et al., 1998). However, this restricts our analysis to T cells that are independent of the 62/63 residues as was first reported by Carson et al. (1997). After immunization, reactive T cells against the 48–61 peptide in the LDA were then examined for their reactivity to HEL and the peptide (Figures 5A and 5B): the T cells that recognized the A conformer reacted with both HEL and the peptide, while the T cells that recognized the B conformer reacted only to peptide. The reactivity of representative T cells identified in a single experiment are shown in Figure 6.

The number of 48–61 reactive T cells was reduced in the two HEL transgenic lines, WT-mHEL and ML-5 (Figure 5A). In four separate experiments, the number of the type B T cells accounted for about 33% of the 48–61 response in the immunized B10.BR mice (Figure 5B). In contrast, 100% of the 48–61-reactive T cells from the WT-mHEL were specific for the type B conformer (Figure 5B). Thus, the tolerance was complete for the A-reactive cells but incomplete for the B-reactive ones (i.e., the frequency of type B–specific T cells was only reduced between 2- and 20-fold when compared to the B10.BR response). Thus, although the level of presentation by APC of the 48–62 family peptides is relatively high in the WT-mHEL mice (3,000–20,000 complexes per APC), T cells recognizing this epitope in the type B conformation escaped tolerance.

As the ML-5 mice had a low level of response following immunization with HEL, we predicted that there would be a T cell response following immunization with the HEL tryptic peptide 46–61. While there was a reduced peptide-specific T cell response in the ML-5 mice, we observed a differential tolerance between the type A– and type B–reactive T cells in that the A-reactive T cells were preferentially affected (Figure 5B).

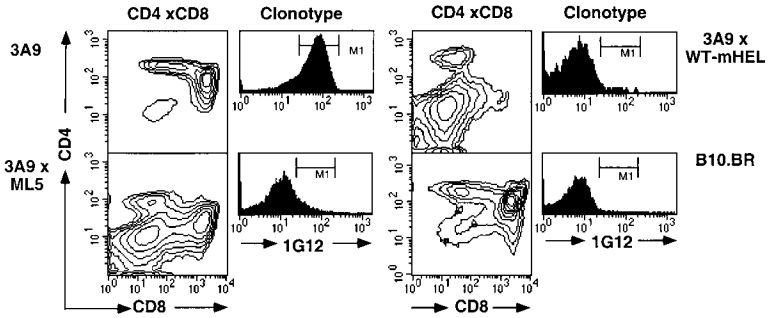

Figure 4. The Analysis of 3A9/HEL Double Transgenic Mice

Thymocytes from mice of 3A9/HEL double-transgenic was analyzed with three-color FACS analysis for CD4, CD8, and 3A9 T cell receptor. CD4 and CD8 expression are displayed in the contour plots on the left. Expression of the 3A9 T cell receptor expression was determined using the 1G12 clonotypic antibody for 3A9. The histograms on the right demonstrate the clonotype expression on $CD4^+CD8^-$ thymocytes. The effective deletion of CD4/CD8 double-positive thymocytes was observed in 3A9 mice crossed to the WT-mHEL mice. In the representative experiment shown here, the total number of thymocytes was dramatically reduced in double-transgenic mice: the 3A9 single-transgenic thymus had 1×10^8 thymocytes; the 3A9/WT-mHEL double-transgenic mice had 1.1×10^6. 3A9/ML-5 double-transgenic mice showed a weaker deleting phenotype, with the persistence of a double-positive population and nearly 10-fold more total thymocytes (1.4×10^7). The expression of clonotype on CD4 single-positive T cells was absent in all double-transgenic mice, indicating that both mice effectively eliminated the 3A9 T cells.

Figure 6A shows the specificity of T cells from B10.BR, ML-5, and WT-mHEL mice identified from LDA. Cells from individual wells were tested on HEL or 48–61 peptide. Shown in this figure is the ratio of the proliferation to HEL (counts per minute [cpm]) to the 48–61 (cpm). We set criteria for type B versus type A distinction based on the response of the T cells to either 5 μM peptide or 50 μM HEL. The density of peptide-MHC complexes generated by 50 μM HEL was much greater than that generated by 5 μM peptide as measured by the AW3.18 monoclonal antibody (data not shown). Clones that incorporated twice as much [^{3}H]thymidine with peptide as with HEL were classified as type B, and those with less than twice as much incorporation were classified as type A. Thus, these criteria defined a type B–reactive T cell as one that preferentially responded to the lower number of complexes formed from peptides compared to HEL.

While the repertoire of T cells in the B10.BR mice demonstrated a spectrum of HEL versus peptide responsiveness, most T cells from ML-5 and WT-mHEL responded better to peptide than HEL. In the T cells cloned from WT-mHEL mice, none of the T cells reacted to 50 μM HEL with [^{3}H]thymidine incorporation 2-fold above background (27 clones analyzed). Figure 6B shows the analysis of representative clones when expanded and then tested for their dose response to HEL and peptide. T cells in Figure 6A, which were classified as type B by their response to 50 μM HEL and 5 μM peptide, all demonstrated type B patterns like those demonstrated in Figure 6B.

Discussion

We addressed here the relationship between peptide presentation and the T cell repertoire in the context of central tolerance. Attempts can be made now to quantitate both parameters, facilitating an objective evaluation of their interdependence. Three main results were obtained. First, we could observe the high sensitivity of central tolerance that operates at the level of a few peptide-MHC complexes per APC. Second, we observed equal tolerance among epitopes expressed at different levels, allowing us to place some limits on the number required to see such an effect. Third, we have identified the escape from negative selection of particular sets of self-peptide-reactive T cells.

Concerning the sensitivity of negative selection, the WT-mHEL transgenic mice were extremely effective in inducing complete tolerance to all HEL epitopes (except to those reflected in the type B conformers). We calculated that the dominant expressed peptide, 48–62, was present in thousands of copies per APC, a number that is much higher than required for negative selection. Of interest, however, were the results that demonstrated partial tolerance in the ML-5 mice. The APC from ML-5 mice only expressed ~100 complexes of I-A^k-48–62 per APC. As noted in the 3A9 transgenic mice crossed to ML-5, this level was sufficient to entirely delete the expression of the 3A9 clonotype. The differences in tolerance between ML-5 and the 3A9/ML-5 may have to do with the affinity of the TCR. The 3A9 T cell probably represents a high-affinity T cell. The T cells of similar affinity were presumably tolerized in the ML-5 mice, while those that escaped and identified were likely of lower affinity (Yule et al., 1993). But the point to be stressed is that the remaining epitopes of HEL are presented at even lower levels, given their weaker binding strength for I-A^k. We have directly quantitated that the presentation of 31–49 peptide family is at about one-twentieth the amount of 48–62 (Gugasyan et al., 1998). As was demonstrated by the immunization of ML-5 mice with HEL, the T cell response to every peptide was affected equally (there was no differential tolerance). Presumably, the higher affinity T cells specific for all of the epitopes were eliminated. Thus, it follows that the number of peptide-MHC complexes of some epitopes causing negative selection of immature thymocytes are at the single-digit levels. We previously reported that ex vivo negative selection of double-positive thymocytes took place on APC bearing an average of only three complexes of 48–61-I-A^k per APC (Peterson et al., 1999).

The high sensitivity of thymic tolerance is to be expected if this tolerance represents a mechanism to eliminate autoreactive T cells to blood proteins. Practically all proteins that enter the thymus would be expected to tolerize. The same would apply to peptides derived from endogenous proteins expressed in thymic APC. In our examples with HEL, we speculate that to observe differential tolerance, 48–62 would have to be presented at a

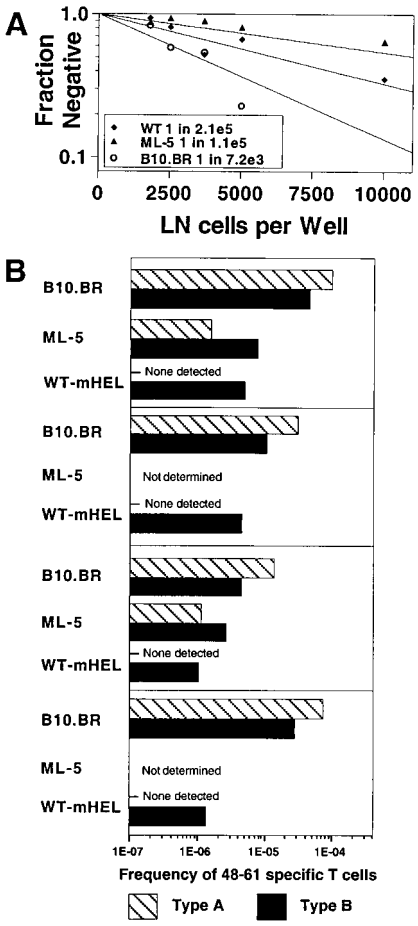

Figure 5. Response to 46–61 Peptide Immunization

(A) The LDA of mice immunized with the 46–61 peptide. B10.BR, WT-mHEL (WT in the figure), and ML-5 mice (ML-5) were immunized with 10 nmols of 46–61 HEL peptide. This figure shows the LDA of growth-positive wells as scored on day 7. These wells were subsequently expanded and tested for their specificity. The precursor frequencies shown in the box represent the actual frequency of specific T cells after correction for the specificity analysis.

(B) The precursor frequency of type A and type B T cells is represented as was described in Figure 2C. The results of four individual experiments are shown. While type A T cells were undetectable in WT-mHEL mice, type B T cells demonstrated only a 2- to 20-fold reduction compared to the B10.BR mice. The ML-5 mice demonstrated a greater reduction in type A precursor frequency than type B precursor frequency when compared to the B10.BR mice.

very low level where the display of 31–49 was practically absent. In this context, the 20-fold difference in the abundance of these two epitopes represents a narrow range; to observe differential tolerance between two epitopes probably requires that these differences be much greater, i.e., 100- to 1000-fold.

In brief, to escape negative selection, peptide epitopes would have to be absent from thymic APC or expressed at single levels by weak-binding interactions with class II molecules. The point of weakly binding peptides and tolerance was previously addressed by the laboratories of Wraith and Goverman with their studies on the response to myelin basic protein peptides (Fairchild et al., 1993; Liu et al., 1995; Harrington et al., 1998) and by our laboratory in the context of the diabetogenic I-A^{g7} molecule (Carrasco-Marin et al., 1996; Kanagawa et al., 1998). These results indicate that these weak interactions allow for negative selection to be impaired, which was documented in the studies with the ac1–11 epitope of myelin basic protein and in the analysis with I-A^{g7}.

We identified T cells that escaped tolerance. Immunizing the WT-mHEL transgenic mice with peptide revealed a population of T cells specific for the type B conformer of 48–62 (a self-peptide). This proves that there is a population of T cells very specific for the type B conformer of the peptide-MHC complex yet unreactive with the type A conformer. Indeed, this set of T cells was not affected by the extremely high levels of 48–62 peptide present in the WT-mHEL mice (thousands of peptide-MHC complexes/APC). The importance of this population could be profound. They may represent an ignorant population of self-specific T cells that, having no tempering of their response as would be expected for type A–reactive T cells, could proliferate and provide T cell help against self-antigens (Ohashi et al., 1991).

Under what circumstances could these phenomena be significant in autoimmunity? In the case of the T cells reactive with a B conformation that escapes central thymic deletion (i.e., WT-mHEL mice), it could imply that these autoreactive T cells circulate and could be activated if they encounter a peptide-MHC complex in the type B conformation. The activation of the uniquely specific type B T cell population would require unique circumstances akin to inflammation for their activation: (1) the extracellular digestion of self-protein and subsequent loading of APCs (Accapezzato et al., 1998), (2) the release of previously processed peptides from MHC antigens, followed by reloading, or (3) the denaturation or unfolding of a self-protein and subsequent loading onto surface class II antigens. Denatured or digested protein could presumably be found in sites of inflammation.

We described in a previous report a “biochemical margin of safety” whereby T cells that escape negative selection in the thymus cannot be triggered in the periphery by the usual density of self-peptide-MHC complexes displayed by most APCs, but require 100-fold more peptide-MHC complexes (Peterson et al., 1999). Thus, most naive T cells remain dormant, since the threshold number of peptide-MHC complexes in APC for triggering these cells may never be realized. However, a T cell that has been primed by previous encounter with antigen, particularly the unique circumstances we have described for the formation of type B conformations, will modify its threshold of activation. This activation now decreases to much lower numbers the T cell threshold of peptide-MHC complexes needed for triggering. Thus, these T cells can be activated by a low level of self-complexes. We are currently evaluating these possibilities.

Finally, the escape of type B T cells from tolerance should be considered in the analysis of T cells in autoimmunity and when mice are immunized with self-peptide. Indeed, considering our results revealing type B T cells and the results of Schild et al. and Purcell et al., who found that immunizing mice with synthetically derived self-peptides elicited T cells specific for contaminants

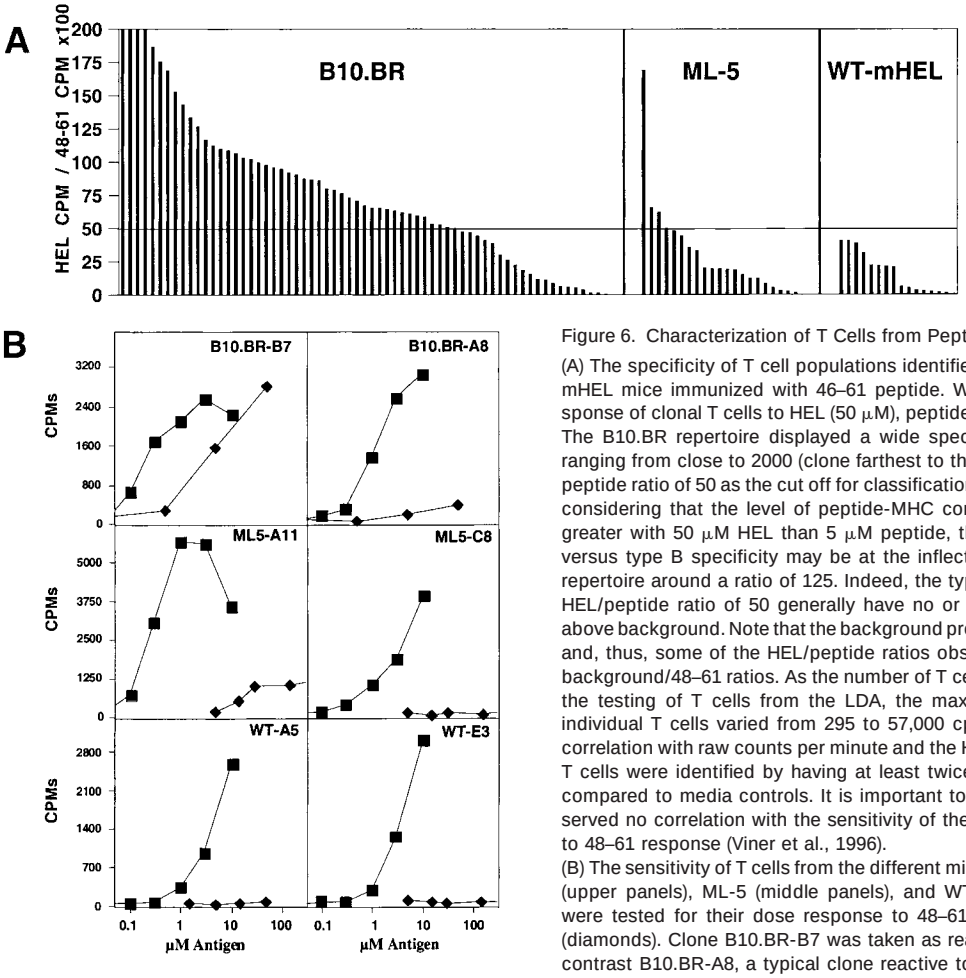

Figure 6. Characterization of T Cells from Peptide-Immunized Mice

(A) The specificity of T cell populations identified in B10.BR, ML-5, and WT-mHEL mice immunized with 46–61 peptide. We compared the relative response of clonal T cells to HEL (50 μM), peptide (5 μM 48–61), or no antigen. The B10.BR repertoire displayed a wide spectrum of HEL/peptide ratios, ranging from close to 2000 (clone farthest to the left) to 0. We chose a HEL/peptide ratio of 50 as the cut off for classification as a type B T cell. However, considering that the level of peptide-MHC complexes per APC is actually greater with 50 μM HEL than 5 μM peptide, the true separation of type A versus type B specificity may be at the inflection observed in the B10.BR repertoire around a ratio of 125. Indeed, the type B T cells found below the HEL/peptide ratio of 50 generally have no or very weak response to HEL above background. Note that the background proliferation was not subtracted and, thus, some of the HEL/peptide ratios observed were the same as the background/48–61 ratios. As the number of T cells was not controlled during the testing of T cells from the LDA, the maximum counts per minute of individual T cells varied from 295 to 57,000 cpm. There was, however, no correlation with raw counts per minute and the HEL/peptide ratio. All specific T cells were identified by having at least twice the proliferation to antigen compared to media controls. It is important to note that we previously observed no correlation with the sensitivity of the T cells and the ratio of HEL to 48–61 response (Viner et al., 1996).

(B) The sensitivity of T cells from the different mice. T cell clones from B10.BR (upper panels), ML-5 (middle panels), and WT-mHEL mice (lower panels) were tested for their dose response to 48–61 peptide (squares) and HEL (diamonds). Clone B10.BR-B7 was taken as reactive to the A conformer. In contrast B10.BR-A8, a typical clone reactive to the B conformer, responds to HEL very weakly at high concentrations. The clones in the middle and lower panels are type B reactive.

of the peptides, all past and future experiments involving such manipulations should be examined critically (Schild et al., 1991; Purcell et al., 1998). Many experiments have been performed in which mice were immunized with peptides, and those in which T cells were unresponsive to intact antigen may need to be revisited (Gammon and Sercarz, 1989; Cibotti et al., 1992; Mamula, 1993; Moudgil and Sercarz, 1993; Cabaniols et al., 1994; Rider et al., 1996; Barlow et al., 1998; Targoni and Lehman, 1998). Indeed, studies like those of Wen et al. may already suggest that type B–specific T cells can be involved in an autoimmune process (Wen et al., 1998).

Experimental Procedures

Mice

The ML-5 mice were generated by Dr. Christopher Goodnow (Goodnow et al., 1988) and were backcrossed to B10.BR mice. The 3A9 T cell receptor transgenic mice (Ho et al., 1994) were a generous gift from Dr. Mark Davis (Stanford University). The WT-mHEL and A-7 mice were generated using a cDNA gene for chimeric HEL-L^d fusion protein (Nelson et al., 1997) under the I-E α promoter (Kouskoff et al., 1993). The construct was injected into B6 oocytes and the transgenic progeny were subsequently backcrossed to the B10.BR strain. All mice were maintained at the Washington University small animal facility. ML-5 mice were screened by serum ELISA for HEL (Yule et al 1993); WT-mHEL mice were screened by FACS analysis of peripheral lymphocytes with anti-HEL antibody F10.6.6. The 3A9 transgenic mice were screened by FACS analysis of lymphocytes using the 1G12 anti-clonotype monoclonal antibody. B10.BR and B10.A(4R) mice were obtained from Jackson Laboratories and bred at the Washington University small animal facility.

Antibodies

Anti-CD4 and anti-V-β 8 were purchased from Pharmingen. Anti-CD8 quantum red (QR) was purchased from Sigma Chemical. The anti-HEL-specific antibody F10.6.6 (Fischmann et al., 1988) was the generous gift of Dr. Roberto Poljak. 1G12 is a monoclonal antibody (IgG1 isotype) specific for the 3A9 T cell receptors. Mice were intraperitoneally immunized biweekly with 1–2 × 10^7 3A9 T cells and boosted 3 days prior to fusion by intravenous injection. Hybridomas were made by the standard fusion techniques using splenocytes of the immunized CB.17 mice. Their supernatants were screened for the ability to stimulate the 3A9 T cell hybridoma (Kanagawa, 1988). The AW3.18 monoclonal used for the quantitation of 48–62-I-A^k complexes was previously described (Dadaglio et al., 1997).

Presentation of the 48–62 Epitope to 3A9 T Cells

Titrating numbers of splenocytes from the HEL transgenic mice were incubated with 1 × 10^4 lymph node cells per well from 3A9 transgenic mice in 96-well U-bottom plates. Proliferation was assessed by incorporation of [^{3}H]thymidine by pulsing the cultures from days 3–4 of the 4 day culture.

Quantitation of 48–62 Epitope on APCs Using the AW3.18 Monoclonal Antibody

The antibody was iodinated using the Chloramine T method as described previously (Dadaglio et al., 1997). Splenocytes were T cell depleted by complement fixation with the anti-Thy-1 antibody (AT83A). Thy-1 depleted splenocytes (2×10^7) from the HEL transgenic or B10.BR mice were incubated with titrating amounts of labeled antibody for 2 hr at 4°C (in triplicate). The cells were then spun through oil to separate bound from free antibody. Nonspecific background (on B10.BR splenocytes) was subtracted from the WT-mHEL staining. The total number of sites per cell was determined by plotting the nanograms of bound antibody as a function of the nanograms of antibody added to each reaction. The nonlinear regression was performed fitting the data to the equation $Y = Bmax*X/(Kd + X)$, a built in equation on the computer program Graphpad Prism. The data fit the curve shown in Figure 1B with an R^2 value of 0.984. Once the amount of antibody bound at saturation was determined, the sites per cell were then calculated.

Examination of 3A9 Thymocytes Crossed to HEL Transgenic Mice

3A9 mice were crossed onto the various HEL transgenic mice. All double-transgenic mice were F1 crosses. Thymi were removed and counted by hemocytometer. Thymi were stained for CD4 (FITC), CD8 (Quantum red, Sigma), and 1G12-biotin followed by Neuralite-avidin-PE secondary. As a control CD3 expression was evaluated on all mature T cells.

Limiting Dilution Analysis

LDA was done as previously reported (Kelso and Glasebrook, 1984; Kanagawa et al., 1998) with some important modifications. Mice were immunized with 10 nmols of either HEL or peptide emulsified in CFA (Difco Laboratories) subcutaneously (five mice per group). Seven days after immunization, the draining lymph nodes were removed and disrupted by pushing them through a 70 μm nytex mesh and pooled. Cells were counted and placed in limiting numbers per well into 96-well U-bottom plates. They were stimulated with 7.5×10^5 irradiated splenocytes (3000 rads), 50 U/ml recombinant murine IL-2, and antigen. For the cloning of HEL-specific T cells, the spleens of WT-mHEL transgenic mice were used as a source of APCs expressing high levels of HEL peptides. T cells from mice immunized with peptide were cloned on 1 μM peptide.

Wells were microscopically examined for cell growth after 7 days. The frequency of growing cells was then determined using Poisson statistics as described by Good et al. (1983). The data was fitted to the following equation as a user-defined nonlinear equation using the computer program Graphpad Prism: $[Y = e^{-AX}]$, where Y is the fraction of wells that are negative, A is the frequency of responding T cells, and X is the average number of LN cells per well.

Growth-positive wells were expanded with either 5×10^5 APCs, antigen and IL-2, or IL-2 alone until there were sufficient numbers of T cells per well for specificity testing. The fraction of antigen-specific cells out of the total number of growth-positive wells was then used to determine the frequency of antigen-specific cells.

To analyze their peptide specificity, clonal wells from the LDA were expanded until sufficient T cells were present to divide into 3–8 well assays. T cells were then stimulated by 5×10^5 APCs plus the relevant antigen, in the absence of exogenous IL-2 in U-bottom 96-well plates. Each point was done with a single well per antigen. T cells were subsequently scored visually for growth and pulsed for the last 12 hr of a 72 hr culture with [^{3}H]thymidine. Antigen-specific T cells were identified by having thymidine incorporation greater than 2-fold above background, and type B T cells were identified by having greater than 2-fold greater counts for 5 μM peptide than for 50 μM HEL. The counts per minute for HEL divided by the counts per minute for 48–61 × 100 was then graphed in Figure 6A. The data was then sorted in descending order and plotted on the histograms as shown. The T cell response from HEL-immunized mice were tested against peptides 18–33, 31–49, 48–63, and 115–129. To determine the specificity of the T cells to peptides bound to I-A^k or I-E^k, the T cells were tested for response to HEL on B10.BR and B10A(4R) splenocytes.

Peptides and Peptide Binding Studies

Peptides were synthesized on a Synergy 432A peptide synthesizer (Applied Biosystems) or Symphony/Multiplex peptide synthesizer (Protein Technologies). The peptides were made using the F-moc chemistry. Peptides were purified by reverse-phase HPLC and the quality of each peptide was determined by mass spectrometry. The relative binding strength of each peptide was determined as described previously (Nelson et al., 1996). Briefly, purified I-A^k from detergent-lysed APCs was incubated with a ^{125}I-labeled standard peptide (sequence YEDYGILQINSR) in the presence or the absence of the peptides to be tested. The amount of bound standard peptide was then determined by standard γ counter following the separation of class II and free peptide on Bio-Spin-6 size exclusion chromatography columns (Bio-Rad Laboratories). The concentrations of each peptide required to inhibit 50% of the maximum binding was compared to the concentration of the cold standard peptide required to inhibit 50% of the maximum binding (RIC^{-1} = Concentration-$50\%_{test}$/ Concentration-$50\%_{reference}$).

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